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Stimulated biosynthesis of delphinidin-related anthocyanins in tea shoots reducing the quality of green tea in summer

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Abstract

BACKGROUND: Greater proportions of purple tea buds and leaves usually appear in the summer, which seriously affects the color and taste quality of green tea products, yet the metabolism of purple tea shoots in summer remains unclear. Here, the metabolomic profiles and gene expression of related flavonoid metabolic pathways in the purple and normal green shoots of 'Longjing 43', and the quality of green tea made with these two phenotypes, were analyzed and compared.

RESULTS: Differential metabolites identified using high-performance liquid chromatography–Orbitrap/mass spectrometry indicated that anthocyanin biosynthesis in purple leaves was enriched, with higher levels of anthocyanidins (delphinidin-hexose-coumaroyl showed the greatest increase), proanthocyanidins (oligomers of catechins) and kaempferol glycoside. Expression patterns of the genes *ANR*, *ANS*, *FLS*, *LAR*, *C4H*, *PAL*, *CHI*, *CHS* and *DFR* revealed that the metabolism of anthocyanin is positively regulated by high temperature and/or light levels in summer. Gas chromatography–mass spectrometry results showed that, in purple tea shoots, the metabolism of carbohydrates was enriched whereas that of amino acids was diminished, while their mannose, fructose, D-galactose, sorbose and D-glucose contents were more than double those found in green leaves. A sensory evaluation confirmed that a greater quantity of purple shoots had a greater negative impact on green tea quality because of a bitter taste and dark color (leaves and infusions were tested).

CONCLUSIONS: These results highlight the need for and possibility of improving commercial tea quality via cultivation that controls the temperature or light of tea gardens during the summer.

Supporting information may be found in the online version of this article.

Keywords: anthocyanin; *Camellia sinensis*; metabolism; purple tea shoots; summer

INTRODUCTION

Leaf color is an important genetic trait of tea (*Camellia sinensis*) plants that can respond to a suite of biotic and abiotic factors in the growing environment.[1] Generally, tea bud leaves are green but sometimes they also appear as reddish purple bud leaves; their amount and color intensity, which are closely linked to commercial tea varieties and the growing season, can greatly influence the quality of a tea.[2] 'Longjing 43', a popular tea clone, is well known for producing Longjing tea – a well-known Chinese green tea that is widely consumed. In the summer, tea shoots in a Longjing 43 tea garden include many purple bud leaves, and the green tea produced from them not only lacks a green color but more importantly tastes bitter, thus greatly limiting the production of green tea in summer.

High anthocyanin accumulation is considered a major cause of tea bud leaves' purple appearance.[3,4] In recent years, purple shoot clones and their derived teas have garnered much attention and scientific interest for the potential pharmaceutical properties of their anthocyanins and flavonoid pigments.[4] For example, the anthocyanin composition and the antioxidant activities from a purple leaf-colored tea cultivar, 'Zijuan', have been identified and characterized.[5] To elucidate the molecular basis of color change during the leaf expansion phase in purple-shoot tea plants, Zhou *et al.* analyzed the differential expression of genes in both tender

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and mature leaves during their growth using cDNA-AFLP.[2] Other researchers[6,7] have performed transcriptome analyses using the Illumina sequencing method, which identified many differentially expressed genes in the purple buds and leaf formation of various purple bud sport cultivars. Using proteomics techniques, researchers have also identified some differential proteins associated with the regulation of anthocyanin metabolism in the leaves of 'Zijuan' and 'Wuyiqizhong 18' tea plants.[2,8] Sun *et al.* successfully isolated the R2R3-MYB transcription factor CsAN1, which could regulate and control anthocyanin biosynthesis in *Camellia sinensis*.[9] Moreover, anthocyanins have been reported as potential dietary components for nutritional and management of several health disorders.[4] In sum, the research to date suggests that anthocyanin accumulation in these purple tea varieties is likely due to a tea bud mutant. However, the constituents of anthocyanin accumulation in purple shoots varied among the different varieties tested. More importantly, how anthocyanin metabolism is regulated at the molecular level is far from clear.

Unlike the purple bud sport cultivar, whose tender leaves are purple almost year round, the purple bud leaves of Longjing 43 appear under summer conditions of high temperature and strong light. Anthocyanin biosynthesis in the purple bud sport tea cultivar has been widely explored, but the mechanism by which the plant's growing environment governs the occurrence of purple tea shoots is not fully understood. We do know that anthocyanin synthesis and metabolism are closely correlated with a tea plant's immediate environment, and that light availability and air temperature are the major influential factors.[10,11] Considering the high frequency of occurrence of purple tea shoots in summer, prior studies have focused primarily on investigating the biochemical components involved in this phenomenon, suitability for processing, tea taste characteristics and quality evaluations.[12] Conspicuously absent are investigations of the regulatory mechanism behind this phenomenon. To rectify this uncertainty and better understand the high frequency of occurrence of purple tea shoots in summer, we analyzed the metabolomics and transcript expression of genes encoding enzymes related to flavonoid metabolic pathways for two tea plants differing in leaf color. Building on this, we further explored the potential regulatory mechanisms for the accumulation of anthocyanins in summer in purple tea leaves.

MATERIALS AND METHODS

Plant material

The tea plants (*Camellia sinensis* cv. Longjing 43) were already established at the Tea Research Institute, Chinese Academy of Agricultural Sciences (TRI, CAAS). The purple and normal green young shoots of one bud with two leaves were randomly selected and taken from the same tea plants in summer (on July 18, 2016). They were immediately frozen with liquid nitrogen, and stored at −70 °C in an ultra-refrigerator. This sampling was repeated six times. Freeze-dried samples were ground into powder using a ball mill (M301, Retsch, Germany) prior to further analysis. Tea products were processed with young shoots harvested at the developmental stage of a bud with one expanding young leaf. For the purpose of comparing farmers' production habits, 50% green + 50% purple shoots were sampled. All young shoots were selected and harvested randomly, and then they were processed into green teas using a standardized procedure (including fixing, rolling and drying). Tea samples were stored in a refrigerator at −20 °C.

Sensory evaluation

The quality score of teas was blindly assessed according to a standardized procedure (GB/T 23776-2009). The appearance, taste, aroma and color of infusions, as well as key features of the infused leaves, were assessed and scored by a tasting panel that consisted of five officially certified tea tasters. The infusion process consisted of 3 g of leaf tea samples infused in 150 mL of freshly boiled water for 4 min. The total score per sample was calculated by summing the individual taster scores for the attributes mentioned above first adjusted by weighting factors of 0.25, 0.30, 0.25, 0.10 and 0.10, respectively.

Gas chromatography (GC)-based metabolomics

Extraction of polar primary metabolites was adapted from Mokochinski *et al.*[13] An amount of 200 mg of tea leaf powder was extracted with 1.4 mL of 80% cold methanol containing ribitol as internal standard. After vortexing (10 s) and centrifugation (17 000 × *g*, 10 min), 500 µL of the supernatant was mixed with 375 µL of chloroform and 750 µL of Milli-Q water. Then, 50 µL of the upper (polar) phase was dried by vacuum centrifugation. Automated derivatization of the metabolites, with methoxyamine and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, was performed using a CombiPAL pipetting–autosampler robot (CTC Analytics AG, Zwingen, Switzerland) mounted on top of the GC instrument. After derivatization, a series of alkanes was automatically added to each sample as well. Extracts were analyzed using a GC–time-of-flight mass spectrometry (MS) system comprising an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to a Pegasus III time-of-flight MS instrument (Leco Instruments, Saint Joseph, MI, USA). Chromatographic separation was performed using a capillary column (Agilent DB-5, 30 m × 0.25 mm i.d., 0.25 µm; Santa Clara, CA, USA). Hard ionization was performed at 70 eV.

Liquid chromatography (LC)-based metabolomics

Samples were extracted essentially according to the method proposed by De Vos *et al.*[14] An amount of 25 mg of dry powder was extracted with 1 mL of 75% MeOH acidified with 0.1% formic acid. After vortexing, sonification for 15 min and centrifugation, 2 µL of the resulting supernatant was injected into the LC–MS (Waters Acquity HPLC coupled to a Waters PDA detector and a Thermo Ion Trap-Orbitrap FTMS hybrid MS system). Compounds were separated with a Luna C18 (Phenomenex, 150 × 2 mm i.d., 3 mm; Torrance, USA) column and a gradient of 5 to 75% acetonitrile acidified with 0.1% formic acid, as described by Mokochinski *et al.*[13] The column was kept at 40 °C and the flow rate was 0.19 mL min^{-1}. The PDA detector was set at a wavelength range of 210–600 nm and the Orbitrap FTMS at an *m/z* range of 90–1350 D, in negative electrospray ionization mode and a mass resolution of 60 000 (FWHM). Subsequently MS–MS analysis was performed using data-dependent acquisition in discovery mode, enabling the mass spectrometer to select the three most intense ions per full scan for fragmentation up to MS3.

Untargeted data processing

For the metabolomics platform the raw data files were processed in an unbiased manner using the dedicated Metalign-MSClust workflow. Metalign software[15] was first used for baseline correction, peak picking and alignment of all mass signals across all samples. The resulting lists with peak intensities (peak heights) of mass signals were filtered for mass features present in at least four samples.

Absent peaks, i.e. with a signal-to-noise ratio < 3, were replaced by random values between 40 and 60% of the local noise input by Metalign. The peak lists were subsequently subjected to MSClust software,[16] in order to cluster mass signals originating from the same metabolite (including, for example, isotopes, adducts and fragments) based on their corresponding retention time and intensity pattern across all samples.

Multivariate analysis and statistics

The preprocessed metabolite intensity data were introduced to SIMCA-P V 13.0 (demo, Umetrics, Sweden) for principal component analysis and partial least squares discriminant analysis after log-transformation and Pareto scaling. Univariate statistical analyses were performed using R (http://www.r-project.org/). Statistically significant differences in mean values were tested using one-way ANOVA with a multiple correlation test using false discovery rate estimation. A Tukey *post hoc* test was applied for comparison of multiple groups. The differences were considered to be significant when $P < 0.05$.

Identification of selected metabolites

The annotation of differentially accumulating metabolites (top 20) was accomplished by matching the extracted data from the chromatograms with available metabolite libraries. In case of LC–MS, observed retention time, accurate mass, isotopic composition, UV spectra and MS–MS information were manually matched with publicly accessible (KNApSAcK, METLIN and MassBank) and in-house metabolite databases obtained from previous studies on tea and other plant species.[17–19] For GC–MS data, annotation was based on comparing both the spectra and the retention index to standard compounds previously analyzed on the same system, the mass spectral database of the Max Planck Institute, Gölm, Germany[20] and NIST.

Quantitative determination of chlorophylls, carotenoids and catechin

An amount of 50 mg of each tea powder sample was extracted with Tris-buffer/MeOH/chloroform (with 0.1% butylated hydroxytoluene (BHT) as antioxidant and Sudan 1 as internal standard) as recently described by Mokochinski *et al.*[13] The samples were washed twice with chloroform. After combining and drying the chloroform phases, the lipid-soluble compounds were taken up in 0.5 mL of ethyl acetate (+0.1% BHT). An amount of 10 µL was injected into the high-performance liquid chromatography (HPLC) system (Waters Alliance e2695, Milford, MA, USA) coupled to a photodiode array detector (Waters 996, Milford, MA, USA) which enabled the recording of the absorption spectra of eluting compounds at 240–700 nm. Chlorophyll a and chlorophyll b were identified based on comparisons of retention time and absorption spectra with authentic standards. External calibration curves were constructed to enable quantification of compounds after correction for variation in the internal standard (Sudan 1). Waters Empower 3 software (Waters, Milford, MA, USA) was used for raw data processing. Catechins were also quantified using HPLC, and the separations were performed using a C18 reverse-phase column (250 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) as described by Zhang *et al.*[21]

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated with an RNAplant_plus kit (Tiangen, China). The cDNA was synthesized with a PrimeScript™ RT reagent kit (TaKaRa) and qRT-PCR was performed with an Applied Biosystems 7300 machine (Carlsbad, USA). Primer pairs used for qRT-PCR are shown in Table S1 and *GAPDH* was used as the reference gene. For each target gene, triplicate reactions were performed. Relative transcript levels were then calculated against those of the internal control (*GAPDH*) using the formula $2^{-\Delta\Delta Ct}$. All data shown are expressed as the mean ± SD ($n = 3$).

RESULTS

Variation in leaf color and pigment content in tea shoots

In the summer, when the tea garden is productive, tea shoots of Longjing 43 produced two distinguishable leaf color phenotypes: purple and green (Fig. 1). Although in the same leaf position, these two leaf color phenotypes showed significant differences. Their contents of chlorophylls and carotenoids were also markedly different. Except for the vio/neo content, the concentrations of lutein, chlorophyll a, chlorophyll b, zeaxanthin and carotene in purple shoots were all lower than those in normal green shoots (Table 1).

Metabolomic profiling of purple and green tea shoots

From the HPLC–Orbitrap data, 5956 detection signals were obtained after an extraction and filter treatment, and 61.82% of them (3682 detection signals) could be classed into 513 clusters, with each representing a metabolite. The heatmap (Fig. S1) and principal component analysis score plot (Fig. S2) showed clear separations between the purple and the green leaves. In the purple samples, 142 metabolite levels were raised and 105 metabolite levels were lowered. We performed a *t*-test ($P < 0.01$) and fold analysis (<0.83 or >1.2) to clarify which metabolites were significantly affected by leaf color change (Table 2). A total of 24 differential metabolites were identified; they were mainly responsible for flavonoid biosynthesis and phenylalanine metabolism, and included flavonol glycosides and anthocyanidins. The purple leaves contained a higher level of anthocyanidins, proanthocyanidins (i.e. oligomers of catechins) and monohydroxyflavonoids – mainly as kaempferol-based acylated tetraglycoside – but lower levels of myricetrin and quercetin glycoside than found in green leaves. Among these metabolic products, the delphinidin-hexose-coumaroyl level was augmented the most.

Anthocyanin spectroscopic analysis

To further verify the putative role of purple flavonoid substances in the leaf color change of tea plants, their spectral data were analyzed in depth. We found that the chromatograms of the two groups of leaf samples displayed significant differences at 530 nm (Fig. 2). There were five absorption peaks (A–E) clearly visible, and their corresponding mass spectrometric information is summarized in Table 3. The Put IDs of peaks A and B are unknown; those of peaks C–E were identified, respectively, as proanthocyanidin III, delphinidin-hexose-coumaroyl and proanthocyanidin (catechin/epicatechin conjugate).

Gene expression analysis of flavonoid metabolism

To further verify the change in flavonoid(s) metabolic pathways based on gene expression levels, the relative expression level of major genes known to be involved in flavonoid metabolism were detected using qRT-PCR. The results showed that the upstream *PAL* gene, and the downstream assimilation *ANR* gene and the *ANS*

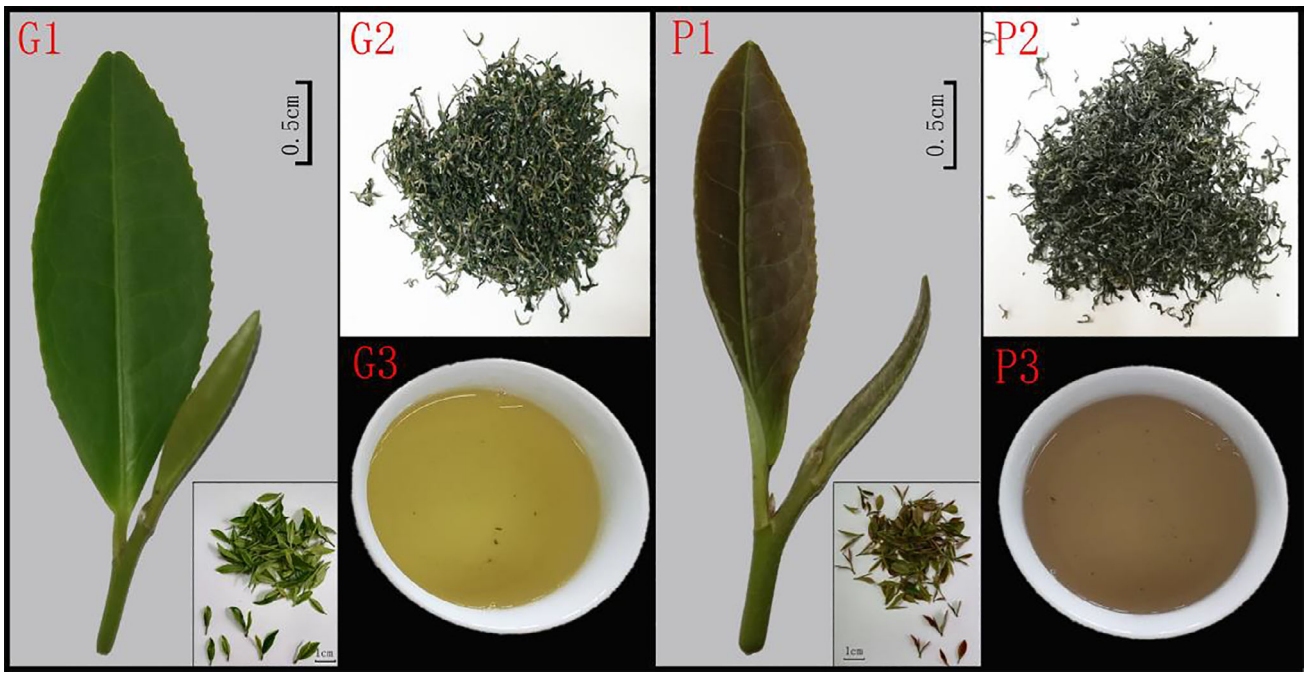

Figure 1. Phenotypes of purple and green young shoots of Longjing 43 tea plants and their derived green teas (G1, normal young tea shoots and infused tea leaf; G2 and G3, normal green tea and tea liquid; P1, purple young tea shoots and infused tea leaf; P2 and P3, purple green tea and tea liquid).

Table 1. Mean (±SD) concentrations of the main catechins and of flavonol glycoside, chlorophyll, carotenoid and tocopherol in the young shoots of purple and green leaves

Name	Green (G) leaves	Purple (P) leaves
Catechins (mg g^{-1} fresh weight)		
Catechin	0.11 ± 0.01	0.10 ± 0.01
Catechin gallate	0.02 ± 0.01	0.02 ± 0.01
Epigallocatechin	0.67 ± 0.03	0.74 ± 0.03
Epicatechin	2.07 ± 0.04	2.00 ± 0.06
Epigallocatechin gallate	10.7 ± 0.20b	12.68 ± 0.21a
Epicatechin gallate	5.84 ± 0.12b	8.17 ± 0.32a
Gallocatechin	0.28 ± 0.01b	0.39 ± 0.01a
Gallocatechin gallate	0.40 ± 0.13b	0.88 ± 0.02a
Flavonol glycoside (mg g^{-1} fresh weight)		
Q-Rut	764.24 ± 138.43	689.38 ± 239.40
Q-RhGa	22.55 ± 2.56	26.16 ± 4.22
K-Glu	22.85 ± 1.14b	31.21 ± 3.70a
Tocopherol (mg g^{-1} dry weight)		
d-Tocopherol	3.18 ± 3.18	2.42 ± 0.85
g-Tocopherol	24.09 ± 3.01a	17.52 ± 2.21b
b-Tocopherol	0.72 ± 0.13	0.42 ± 0.23
a-Tocopherol	21.76 ± 2.2	24.08 ± 2.5
Carotene (μg mL^{-1} fresh weight)		
Vio/Neo	6.18 ± 0.85	6.33 ± 0.55
Lutein	17.14 ± 2.21a	11.18 ± 2.01b
Zeaxanthin	1.13 ± 0.13a	0.65 ± 0.23b
β-Carotene	2.67 ± 0.20a	1.60 ± 0.30b
α-Carotene	1.23 ± 0.20a	0.72 ± 0.10b
Chlorophyll (μg mL^{-1} fresh weight)		
Chlorophyll a	132.64 ± 19.24a	92.97 ± 14.23b
Chlorophyll b	49.99 ± 9.03a	33.19 ± 7.02b

Different letters within a row indicate values that are significantly different at $P < 0.05$.

gene of flavonoid metabolism were all up regulated, especially that of *FLS* whose expression level in purple leaves was 3.2-fold higher than that in green leaves (Fig. 3).

Primary metabolism and quality-related compound changes between purple and green leaves

To further analyze the influence of leaf color change caused by anthocyanin metabolism on tea quality, key components – amino acids, carbohydrates, organic acids and catechins – were quantitatively determined using GC and LC techniques. The GC–MS results showed that the amino acid content in purple leaves declined significantly (Table 4), such that their levels of L-theanine, glutamic acid and alanine were, respectively, 0.22, 0.48 and 0.51 times those found in green leaves. The organic acid content in purple shoots was also significantly reduced, namely citric acid, malic acid, alanine, α-ketoglutaric acid, propanoic acid, phosphoenolpyruvate and gallic acid. However, the carbohydrate content in purple leaves was significantly increased, such that the mannose, fructose, D-galactose, sorbose and D-glucose concentrations were more than two times those found in green leaves (xylose showed a 1.61-fold increase). Table 1 presents the differences in catechin and major flavonol glycoside contents. Epigallocatechin gallate, epicatechin gallate, gallocatechin and gallocatechin gallate contents were significantly increased in the purple leaves, in which K-Glu was also markedly increased, while the Q-Rut and Q-RhGa contents were unchanged between the two leaf colors.

The vitamin content in the tea shoots differing in leaf color showed significant differences between them (Table 1). The contents of d-tocopherol, g-tocopherol and b-tocopherol in purple leaves were significantly lower than those in green leaves, especially that of g-tocopherol, which was reduced from 25 to 18 mg g^{-1}. By contrast, the a-tocopherol content in purple leaves increased by 10% compared with that in green leaves.

Table 2. Alterations of intracellular metabolites induced by leaf color change

Put ID	P mean	G mean	*P* value (*t*-test)	Log2 (P/G)
Catechin-3-*O*-acetoxybenzoate	12.9	14.0	0.0002	−1.13
Delphinidin-hexose-coumaroyl	18.6	15.9	<0.001	2.74
Dihydrokaempferol-hexose or eriodictyol chalcone-hexose	17.6	16.2	<0.001	1.42
Gallocatechin catechingallate I	22.3	21.4	0.0001	0.91
Gallocatechin catechingallate II	21.1	20.5	0.0001	0.67
Gallocatechin-3-*O*-(3″-*O*-methyl)gallate	21.0	21.8	0.0001	−0.81
Geranylated catechin ((2*R*,3*R*)-pentahydroxy-6-[6-hydroxy-3,7-dimethyl-2(*E*),7-octadienyl] flavanone)	14.8	16.2	0.0004	−1.43
Kaempferol 3-*O*-(2G-*p*-coumaroyl(*cis*)-3G-*O*-b-L-arabinosyl-3*R*-*O*-b-D-glucosylrutinoside	15.7	15.1	0.0066	0.54
Kaempferol-based acylated tetraglycoside I	17.7	16.1	0.0002	1.53
Kaempferol-based acylated tetraglycoside II	19.1	18.3	0.0004	0.86
Kaempferol-based acylated triglycoside I	15.0	12.5	<0.001	2.46
Kaempferol-based acylated triglycoside II	17.0	15.8	0.0002	1.14
Kaempferol-3-*O*-(glucoside-1,6-*O*-rhamnoside)-4′-*O*-rhamnoside	17.8	16.8	<0.001	1.02
Kaempferol-3-*O*-galloylhexoside	17.4	16.4	0.0001	1.03
Manniflavanone isomer	14.6	14.0	0.0008	0.64
Naringenin chalcone-hexose I	18.6	17.9	0.0001	0.68
Naringenin chalcone-hexose II	15.6	15.1	0.0040	0.55
Proanthocyanidin I	18.3	17.3	0.0007	0.90
Proanthocyanidin II	21.8	21.2	0.0002	0.59
Prodelphinidin	16.8	15.8	0.0002	1.02
Prodelphinidin-*O*-gallate I	21.6	20.6	0.0001	0.97
Prodelphinidin-*O*-gallate II	16.1	15.5	0.0003	0.58
Quercetin-3-*O*-glucoside	19.3	20.0	<0.001	−0.64
Quercetin-3-*O*-rhamnoside	15.1	16.2	0.0001	−1.05

P, purple tea leaves; G, green tea leaves; P/G, ratio of purple to green tea plants.

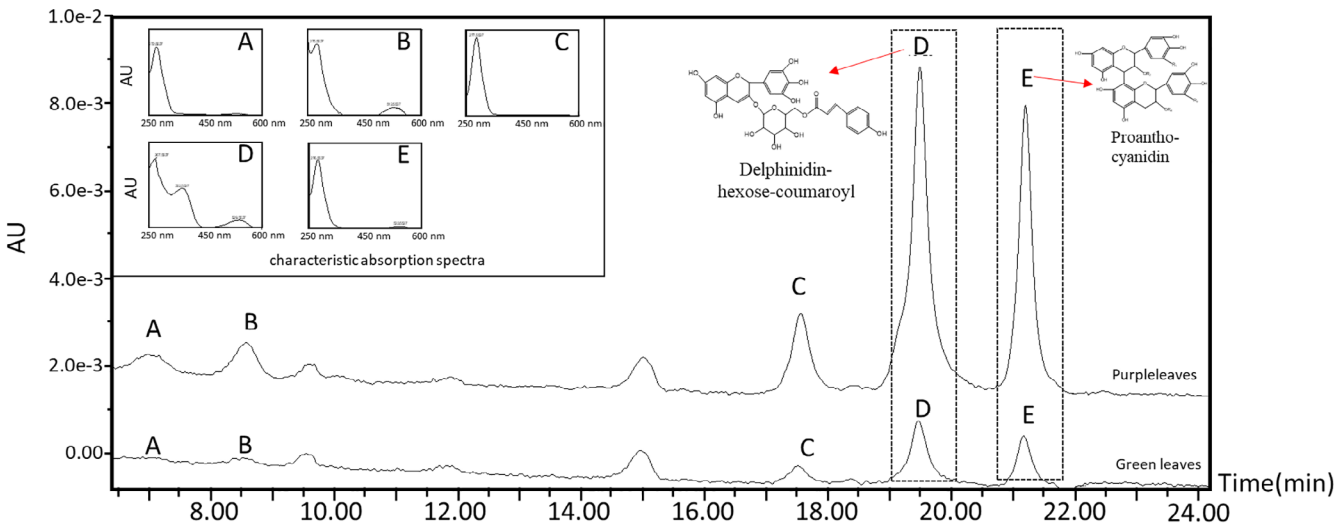

Figure 2. Chromatograms at 530 nm analyzed using HPLC with the spectra (inset) of the main differently accumulated compounds.

Sensory evaluations

The scores for the appearance, taste and aroma of green tea derived from purple buds and leaves were significantly lower than those from green tea made using 50% purple bud and leaf composition, and both their sensory scores were below that obtained for normal green tea (Table 5). The color of green tea made from purple shoots is dark, while that using 50% of purple shoots looks mixed of light and dark; neither could meet the requirement of high-quality green tea. Compared with the normal quality expected of green tea products, purple green tea had a more bitter taste, and looked darker.

DISCUSSION

Flavonoid biosynthesis in purple tea shoots

Secondary metabolism is widely understood to play a critical role in how plants respond to environmental stresses.[22] Flavonoids are the most important secondary metabolites found in the tea plant,

Table 3. Alterations of metabolites in the chromatogram at 530 nm induced by leaf color change

Clusters	Np	RT (min)	MZ	λ_{max} (nm)	Put ID	Log (P/G) fold AU	MI
A	2	6.88	475.0780	273 522	Unknown	2.81	1.63
B	2	8.47	411.0235	276 513	Unknown	3.13	0.56
C	5	17.47	731.1518	277 534	Proanthocyanidin III	2.11	0.59
D	6	19.40	609.1249	349 530	Delphinidin-hexose-coumaroyl	2.28	2.74
E	6	21.11	884.1758	278 522	Catechin/epicatechin conjugate	2.28	0.11

Np, number of peaks; RT, retention time; MZ, mass; P/G, purple/green tea plants; AU, absorbance; MI, mass intensity.

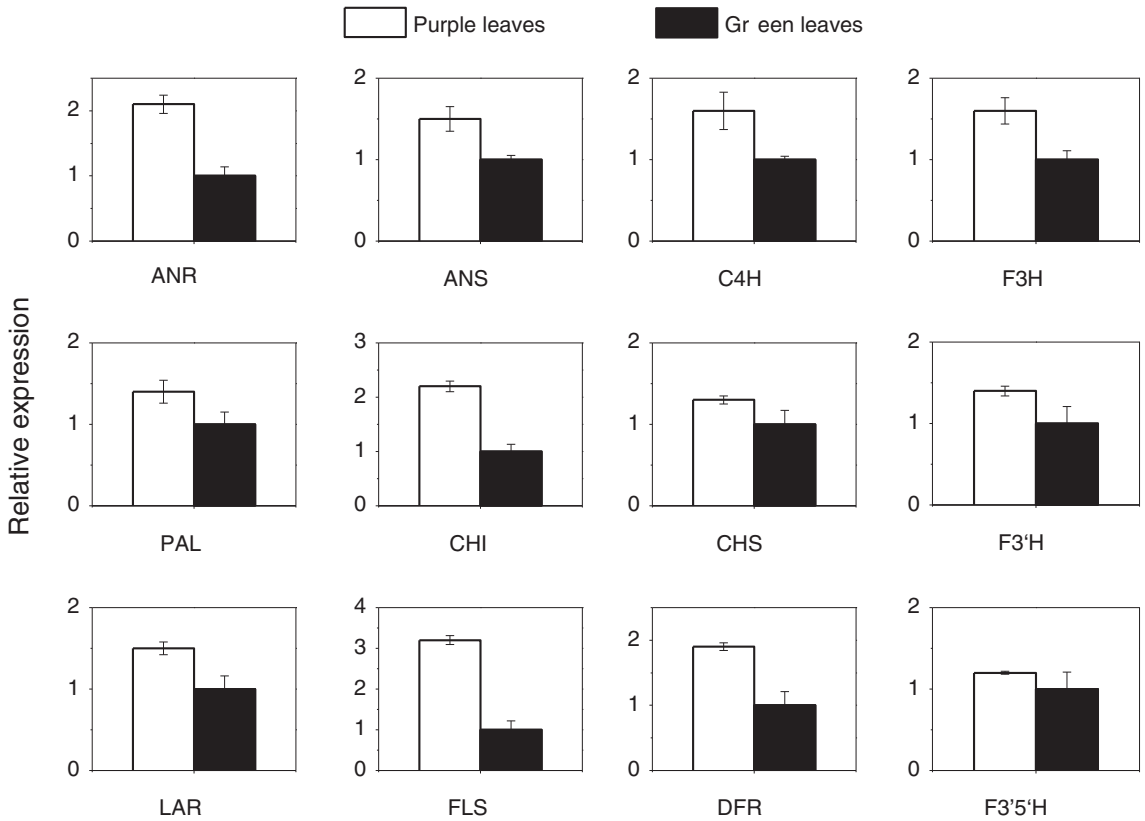

Figure 3. Relative expression of genes related to flavonoid metabolism. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamate 4-hydroxylase; F3′H, flavonoid-3′-hydroxylase; PAL, phenylalanine ammonia-lyase; CHI, chalcone isomerase; CHS, chalcone synthase; F3H, flavonoid-3-hydroxylase; LAR, leucoanthocyanidin reductase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; F3′5′H, flavonoid-3′,5′-hydroxylase. Bars are means ± SD, $n = 3$.

accounting for 18–36% of its dry weight, mainly in the form of catechin, anthocyanins and flavonols. In our experimental study, flavonoid compounds in the two kinds of phenotypic tea shoots were comparatively analyzed via high-resolution LC–MS methods. The results showed that flavonoids principally enriched in purple shoots were procyanidins, anthocyanins and flavonol glycosides, such as catechin polymers, delphinidin-hexose-coumaroyl and kaempferol-based acylated triglycoside (Fig. 4).

Anthocyanin occurs widely in the petals and fruit organization of plants, and in the surface cells and lower epidermis of stem leaves.[10,23] For example, delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside is responsible for the purple skin color of the eggplant fruit,[24] and delphinidin-3-*O*-(6-*p*-coumaroyl)glucoside can be found in red grape cultivars.[25] In our study, delphinidin-hexose-coumaroyl and proanthocyanidins (oligomers of catechins) were identified as the main compositional color compounds in the purple shoots of Longjing 43 tea plants. The former belongs to the anthocyanin glucoside group, which is derived from dihydromyricetin, and its predominance is consistent with previous work on purple peel color mutant of eggplant,[24] but disagrees with findings for the purple leaf color mutant of tea varieties.[5] Hence, the regulation mechanism in Longjing 43 as induced by its environment differs from that in its mutant.

Moreover, anthocyanin color always changes with pH, which provides plants with a range of bright and vivid colors. The altered chemical structure that occurs in response to changes in pH is the reason why anthocyanins are often used as pH indicators, since they readily change from red in acids to blue in bases. The higher-content organic acid compounds in the purple shoots suggested that the pH values in purple and green leaves are

Table 4. Alterations of intercellular metabolites induced by leaf color change

Put ID	P mean	G mean	*P* (*t*-test)	Log2 (P/G)
Amino acid				
Glycine	0.03	0.05	0.03	−0.02
Asparagine	0.03	0.05	0.05	−0.02
Aspartic acid	0.35	0.56	0.01	−0.22
Glutamic acid	1.62	2.54	0.01	−0.92
L-Theanine	0.04	0.19	<0.01	−0.15
Serine	0.32	0.61	<0.01	−0.28
L-Proline	1.14	1.76	<0.01	−0.62
L-Threonine	0.08	0.11	0.02	−0.04
Glycine	0.03	0.05	0.03	−0.02
Asparagine	0.03	0.05	0.05	−0.02
Carbohydrate				
D-Galactose	2.24	0.85	<0.01	1.40
D-Glucose	14.65	5.27	<0.01	9.39
Sorbose	6.08	2.28	<0.01	3.80
β-D-Fructofuranose	0.01	0.02	<0.01	−0.01
Xylose	0.23	0.14	0.01	0.09
Fructose	3.94	1.55	0.01	2.39
Mannose	0.04	0.02	0.01	0.02
Organic acid				
Citric acid	0.85	1.28	<0.01	−0.43
Malic acid	2.96	4.08	<0.01	−1.13
Alanine	0.10	0.19	<0.01	−0.09
α-Ketoglutaric acid	0.04	0.08	<0.01	−0.04
Propanoic acid	0.05	0.08	<0.01	−0.04
Phosphoenolpyruvate	1.28	1.91	<0.01	−0.63
Gallic acid	0.99	0.67	<0.01	0.32

P, purple tea leaves; G, green tea leaves; P/G, ratio of purple and green tea plants.

obviously different. The proanthocyanidins, however, are colorless compounds, and those identified in our study may largely depend on pH changes. Nevertheless, the relationship between the color of anthocyanins and their pH environment requires further study in tea shoots.

Numerous studies show that the metabolism of flavonoids is susceptible to the effects of plant genotype,[26] nutrition[27] and environment.[21] We found that proanthocyanidins – the combination of different numbers of catechin or epicatechin molecules – were highly accumulated in the purple tea shoots. However, this finding is not consistent with that reported for Zijuan tea.[3] This indicates that the causal mechanism behind leaf color change in Longjing 43 is unlike that based on mutations in Zijuan. Thus, an external source of regulation, via ambient temperature and/or light, could be operating in Longjing 43 (but not in Zijuan). Light is reportedly the main factor acting to regulate anthocyanin metabolism[28,29]; for example, Agati *et al.* reported that strong direct light can significantly increase the content of polyphenols in *L. vulgare* leaves.[28] Meanwhile, high temperature has been suggested to promote the biosynthesis of flavonoids.[1,30] Therefore, a higher content of tea polyphenols in summer-grown green tea is usually due to the higher temperature and strong light intensity experienced in summer, which could stimulate the tea plants' synthesis of polyphenols. In our experiment, the contents of most catechins, anthocyanins and flavonol glycosides in the purple leaves were significantly increased, and the primary flavonoid biosynthesis genes (*PAL*, *FLS*, *etc.*) were upregulated. These results are likely best explained by the summer season's high temperature and light intensity.

Flavonol glycosides are considered important antioxidants participating in plant photoprotection and light stress responses,[31,32] and the number of B hydroxyl groups in them is directly related to their antioxidant functions.[33,34] In our study, the kaempferol glycoside levels in the purple leaves were significantly increased, whereas the quercetin levels were significantly reduced, relative to the green leaves. This result suggests that the metabolism of flavonol glycosides is regulated by substrate competition; or, more likely, it is related to differences in bioactivity between kaempferol glycosides and quercetin glycosides in response to environmental changes. Another antioxidant, g-tocopherol, occurred at significantly lower concentration in the purple than green leaves, which means they likely also differed considerably in their antioxidant metabolism. Therefore, further investigations of antioxidant function and the role of flavonol glucoside in tea shoots are warranted. Interestingly, as downstream products of dihydromyricetin, the delphinidin glycoside products showed many accumulated cases, while the change in myricetin between the purple and green leaves was not significant. This suggests that the environment regulates the metabolic flow from flavonols to anthocyanins, an interpretation bolstered by the accumulation of proanthocyanidins (downstream products of anthocyanins).

Effect of purple shoots on quality of summer-grown tea

According to the sensory evaluations made, tea quality is based on its appearance, liquid color, aroma, taste and infused leaf. These sensory characteristics of tea are closely related to its biochemical composition, some of which are affected by tea processing, but, overall, the determining factors are the composition and proportion of metabolites in the raw tea materials. Hence, too great a proportion of purple tea shoots would have an obvious negative influence on green tea processing; this concern was confirmed by the sensory evaluation results.

Taste is one of the most important quality characteristics of tea; high-quality green teas have little bitterness. In the present study, both bitterness and astringency are evident in the purple green tea, which dramatically reduces the quality of green tea. Metabolite analysis showed that this bitterness and astringency were closely associated with the content of catechins and anthocyanins and the ratio of polyphenols to amino acids. Put differently, our results indicate that purple tea shoots have a higher anthocyanin content but a lower amino acid content when compared with green tea shoots. Since a low ratio of polyphenols to amino acids in raw tea shoots is necessary to produce high-quality green tea for consumption, it follows that including purple tea shoots negatively affects green tea quality.

Beyond this consideration, amino acids, organic acids and soluble sugars are the next most important components affecting green tea quality. In this experiment, the contents of amino acids and organic acids were markedly different, which has a crucial influence on tea quality. Moreover, the characteristics of tea for drinking, including its color, taste and aroma, are all directly or indirectly associated with its phenolic compounds.[35–37] We found that the flavonoids in purple tea leaves were significantly increased over green leaves, especially the anthocyanins and proanthocyanidins. However, anthocyanin is not the only bitter-tasting substance in tea made to drink, but also confers an unfavorable color to the

Table 5. Sensory evaluation of tea products derived from the purple and green young shoots of Longjing 43 plants

Tea	Features	Appearance	Taste	Aroma	Color of infusion	Features of infused leaves	Total score
Purple	Score	87	80	84	70	86	82.2
	Assessment	Dark green	Strong	Potpourri	Dark, light red	Yellow green	Middle quality
Green	Score	89	85	85	87	86	86.1
	Assessment	Light green	Bitter	Potpourri	Yellow green	Yellow green	Low quality
50% Green + 50% purple	Score	85	82	84	80	86	83.4
	Assessment	Dark green	Bitter	Potpourri	Yellow green	Yellow green	Low quality

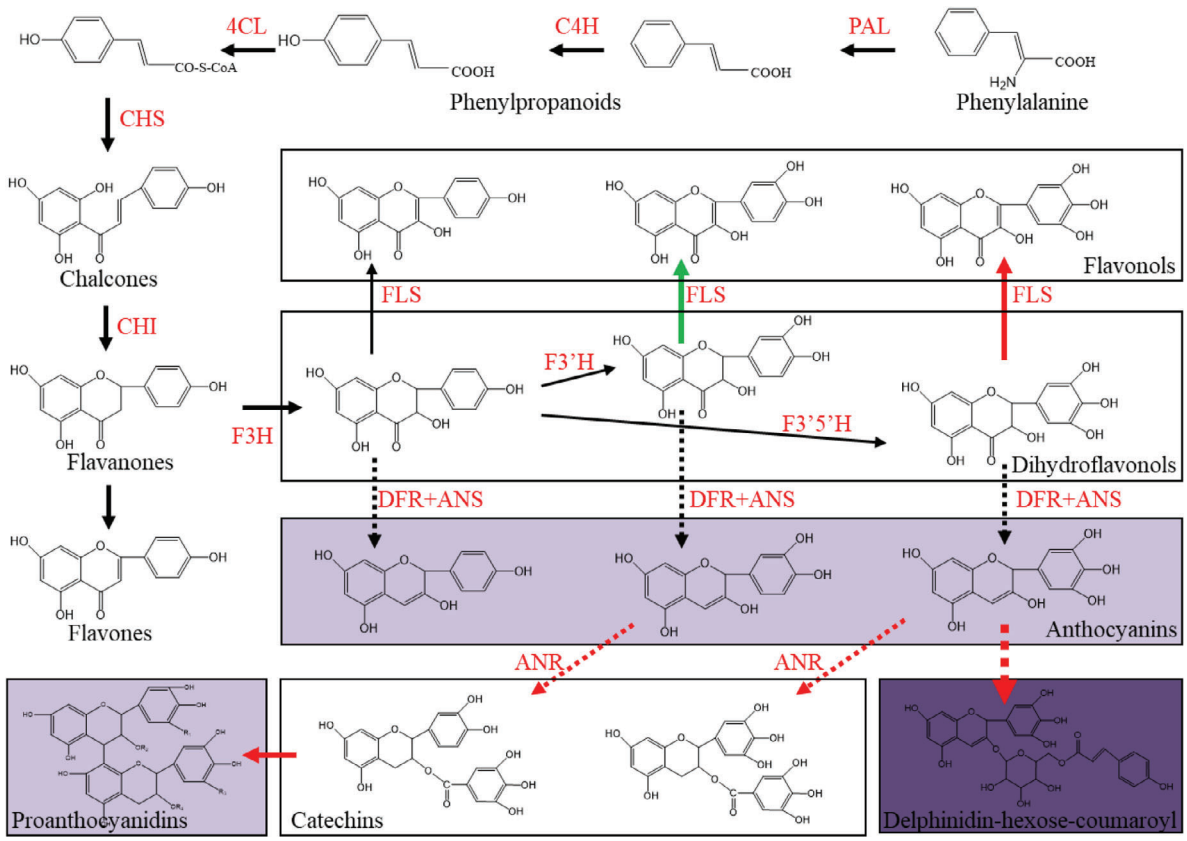

Figure 4. Schematic representation of the phenylpropanoid/flavonoid pathway as affected by leaf color change. The red and green fonts indicate up- and downregulated genes in the purple leaves compared with the green leaves, respectively. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4Cl, 4-coumarate–CoA ligase; CHI, chalcone isomerase; CHS, chalcone synthase; F3′5′H, flavonoid-3′,5′-hydroxylase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-monooxygenase; FLS, flavonol synthase; DFR, dihydroflavonol-4-reductase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase.

quality of green tea. In addition, flavonol glycosides are the main yellow ingredients in tea infusion. In this experiment, the contents of different flavonol glycosides varied substantially, which makes their effects upon tea quality rather complex.

With respect to its color, high-quality green tea should be green and bright. However, the chlorophyll content is lower in purple leaves, and those chemicals with a purple color (at 530 nm, as evident from Table 3) are present at higher concentrations, which makes the color of green tea produced by purple shoots look dark; this appearance further reduces the quality of green tea. The results from the subsequent analysis indicated that the reddish purple color was mainly caused by how much delphinidin-hexose-coumaroyl, chlorophyll and anthocyanin were present. With an increased organic acid content, the ensuing lowered pH of tea liquid would influence the color environment of the anthocyanins. This may be another reason for why the liquid color of green tea made with purple leaves looks reddish purple.

On the contrary, considering the pharmacological activity of anthocyanin,[4] including antioxidant and antiproliferative properties, accumulation of delphinidins in tea shoots in summer may bring more health benefits when those anthocyanins are consumed, although the taste of the tea product may not be as good. Furthermore, we can change the temperature and light environment through horticultural measures (such as shading), so as to promote anthocyanin synthesis and metabolism for health benefits. And we can also adopt opposite horticultural measures to reduce the accumulation of anthocyanins and ultimately improve the quality of tea in summer.

CONCLUSIONS

Through an HPLC–Orbitrap MS metabolomics analysis, we studied the variation in the metabolites between the purple and normal green leaves of the Longjing 43 tea plant. Our results suggested that purple shoots have a negative effect on the drinking quality of green tea since they increased the bitterness of tea liquid and gave it a deep/dark color. Moreover, anthocyanin metabolism was positively regulated via high levels of temperature or light in the summer, when delphinidin glucoside, the main chemical found responsible for the leaf color change, became highly accumulated in leaves.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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